

HIGH PREVALENCE OF VIABLE *TOXOPLASMA GONDII* INFECTION IN MARKET WEIGHT PIGS FROM A FARM IN MASSACHUSETTS

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ABSTRACT: The ingestion of uncooked infected meat is considered important in the epidemiology of *Toxoplasma gondii* infection in humans and little is known of the prevalence of viable *T. gondii* in meat used for human consumption in the United States. In the present study, viable *T. gondii* was isolated from 51 out of 55 pigs destined for human consumption. Hearts and tongues (500 g) from fifty-five 6-mo-old pigs from a farm in Massachusetts were bioassayed for *T. gondii* by feeding them to *T. gondii*-free cats. Feces of these cats were examined for shedding of *T. gondii* oocysts. Fifty-one of 55 cats fed pig tissues each shed 25–810 million *T. gondii* oocysts in their feces. Two of these cats consumed tissues of pigs that were shown to be seronegative with the Sabin–Feldman dye test, the modified agglutination test, and the Western blot. Results indicate that until examination of meat for *T. gondii* infection is implemented in slaughterhouses, all meat should be cooked according to industry guidelines before human consumption.

In the most recent national survey, antibodies to *Toxoplasma gondii* were found in 22.5% of 17,658 serum samples from persons 12 yr or older in the United States (Jones et al., 2001). Among 4,926 women of child-bearing age (15–44 yr), antibodies to *T. gondii* were detected in 15%. These data reinforce the view that the majority of women of child-bearing age in the United States are at risk of transmission of *T. gondii* infection to their fetus if they acquire infection during pregnancy (Remington et al., 1995).

The ingestion of food or water contaminated with *T. gondii* oocysts or the ingestion of infected raw or undercooked meat are the major modes of postnatal transmission of *T. gondii* in humans (Dubey and Beattie, 1988). At present, there is no test to distinguish meat versus oocyst-acquired toxoplasmosis. Beef, poultry, and pork are the 3 major types of meats consumed in the United States (Dubey, 1994). Of these, the prevalence of viable *T. gondii* in beef and poultry has not, as yet, been critically evaluated. In limited studies conducted 20–40 yr ago, *T. gondii* was not isolated from beef (Jacobs et al., 1960; Remington, 1968; Dubey and Beattie, 1988). There is also no information on *T. gondii* infection in commercially raised poultry in the United States. More is known of the prevalence of *T. gondii* in pigs. In 2 studies performed nearly 40 yr ago, *T. gondii* was isolated from diaphragms of 12 of 50 pigs from a slaughterhouse in Maryland (Jacobs et al., 1960) and from 8 of 50 pork loins from grocery stores in California (Remington, 1968). In a large study conducted a decade ago to establish the validity of different serologic tests used to detect *T. gondii* in pigs, viable *T. gondii* was isolated from 170 of 1,000 sows from a sausage plant in Iowa (Dubey, Thulliez, and Powell, 1995; Dubey, Thulliez, Weigel et al., 1995). With respect to human infections, pork from breeder pigs is not important because meat from sows is often frozen and processed (sausages and bologna) and the processing is likely to destroy *T. gondii* (Dubey and Beattie, 1988).

Serological surveys conducted in the past 20 yr indicate the

prevalence of *T. gondii* in pigs is declining (Dubey, 1990; Zimmerman et al., 1990; Smith et al., 1992; Assadi-Rad et al., 1995; Dubey, Weigel et al., 1995; Weigel, Dubey, Siegel, Hoefling et al., 1995; Weigel, Dubey, Siegel, Kitron et al., 1995; Patton et al., 1996; Davies et al., 1998). For example, in market weight pigs tested by modified agglutination test (MAT) in 1 laboratory under identical conditions, seroprevalence in 1983–1984 was 23.9% and 3.1% in 1992 (Dubey et al., 1991; Dubey, Weigel et al., 1995). In a recent study of pigs from North Carolina, antibodies to *T. gondii* were found in only 13 (0.58%) of 2,238 market weight pigs and only 1 of 1,752 pigs raised in confinement had antibodies (Davies et al., 1998). This reduction in *T. gondii* prevalence is attributed to changing management practices and consolidation of pig production into large-scale operations. Although many pigs raised in the United States are in large-scale operations, there are still many small swine farms and the prevalence of *T. gondii* in some of these pigs is high. Recently, antibodies to *T. gondii* were found in 47% of 900 market weight pigs from 4 New England states (Gamble et al., 1999). The objective of the present study was to document the high prevalence of viable *T. gondii* in pigs from 1 of these farms in Massachusetts.

MATERIALS AND METHODS

Naturally infected pigs

Pigs were obtained from a 500 head farrow-to-finish production facility in southeastern Massachusetts. Previous studies on prevalence in the New England states had demonstrated high rates of infection on this farm (Gamble et al., 1999). Fifty-five sows and 2 boars were maintained on dirt lots. Weaned pigs were fed and finished in enclosed buildings on concrete slabs. All animals were fed cooked garbage consisting of restaurant and processed food waste, as well as produce and bakery waste. Feral cats were observed on the premises, and biosecurity and rodent control was minimal. Finished pigs were marketed at approximately 100 kg, generally between 7 and 8 mo of age.

Two lots of 55 market-aged pigs were examined for *T. gondii* infection. The first group of 30 pigs were chosen based on results of previous *T. gondii* testing using blood drawn at the farm. In a second group, 25 pigs were selected at random from the oldest group of finishers and these pigs had not been previously tested for antibodies to *T. gondii*. Before slaughter, venous blood was obtained from all 55 pigs, and hearts (lot 1) and hearts and tongues (lot 2) were collected for bioassay.

Bioassay of pig tissues for *T. gondii* infection

After trimming fat, connective tissue, and epithelia, approximately 50–100 g of myocardium (lot 1) and 100–250 g of myocardium and

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TABLE I. *Toxoplasma gondii* oocysts shedding by cats fed naturally infected pig hearts (lot 1).

Pig no.	<i>T. gondii</i> antibody titer (MAT)	No. of oocysts shed by cats ($\times 10^6$)
1	100	140
2	≥ 500	190
3	≥ 500	525
4	≥ 500	540
5	100	55
6	500	140
7	100	170
8	500	160
9	100	25
10	≥ 500	70
11	100	105
12	≥ 500	270
13	500	216
14	≥ 500	150
15	≥ 500	55
16	100	60
17	500	260
18	≥ 500	180
19	500	445
20	100	160
21	≥ 500	580
22	≥ 500	208
23	≥ 500	810
24	100	440
25	≥ 500	523
26	≥ 500	320
27	≥ 500	110
28	≥ 500	160
29	≥ 500	75
30	≥ 500	70

tongue (lot 2) from each pig were fed individually to 55 cats over a 2- to 3-day period. The cats were 10–12 wk old, raised in isolation from birth, and obtained from Liberty Research (Waverly, New York). Blood was drawn from a jugular vein of each cat 1 wk before feeding porcine tissues and their sera tested for antibodies to *T. gondii* using the MAT, as described by Dubey and Desmonts (1987). The cats used in the present study had never been fed uncooked meat before these experiments and had no demonstrable MAT antibody in a 1:25 dilution of their sera.

Feces of each cat were collected daily, starting from 2 to 3 days after feeding pig tissues, and examined for the presence of *T. gondii* oocysts. The fecal collection continued until cats stopped excreting oocysts. For microscopic examination of oocysts, the entire feces were emulsified with a small volume of water; then ~10 g of the mixture was mixed with ~40 ml of a sucrose solution (specific gravity, 1.18), filtered through a gauze, and centrifuged in a 50-ml tube at 2,000 rpm (~1,200 g) for 10 min (Dubey, 1995). A drop of the float from the meniscus was examined microscopically for oocysts. If oocysts were detected, the entire daily feces were mixed with sucrose solution in 50-ml tubes and centrifuged at 2,000 rpm for 10 min. The entire supernatant (~40 ml) was mixed with 200 ml of water and centrifuged. The supernatant was discarded, the sediment was mixed with water, and all samples from each cat for each day were pooled, centrifuged, and finally suspended in water to make a final volume of 100 ml. Oocysts were counted in the 4 WBC chambers of a hemocytometer. The number of oocysts in 4 WBC chambers was multiplied by $2,500 \times 100$ (total volume). Thus, the sensitivity of counting oocysts was 250,000 per fecal sample.

Oocysts were sporulated at 22 C for 1 wk in 2% sulfuric acid and treated with sodium hydroxide to neutralize the sulfuric acid (Dubey and Beattie, 1988). Neutralized oocysts from each cat were fed to 2

TABLE II. *Toxoplasma gondii* oocysts shedding by cats fed naturally infected pig tissues (lot 2).

Pig no.	<i>T. gondii</i> antibodies in pigs				No. of oocysts shed by cats (×10 ⁶)
	MAT		Dye test	Western blots*	
	Beltsville	Paris			
31	<10	<10	<10	Neg	None
32	40	80	40	Pos	600
33	≥100	ND†	<10	Neg	318
34	10	10	ND	Pos	138
35	≥100	ND	ND	Pos	215
36	40	40	20	Pos	565
37	20	20	40	Pos	500
38	<10	<10	<10	Neg	165
39	<10	<10	<10	Neg	None
40	≥100	ND	ND	Pos	253
41	≥100	ND	ND	Pos	425
42	<10	<10	<10	Neg	None
43	<10	<10	<10	Neg	None
44	≥100	ND	ND	Pos	415
45	20	40	20	Pos	180
46	20	40	40	Pos	330
47	<10	<10	<10	Neg	213
48	≥100	ND	ND	Pos	160
49	≥100	ND	ND	Pos	325
50	≥100	ND	ND	Pos	155
51	≥100	ND	ND	Pos	173
52	≥100	ND	ND	Pos	338
53	≥100	ND	ND	Pos	195
54	≥100	ND	ND	Pos	125
55	≥100	ND	ND	Pos	400

* Pig 1° antisera diluted 1:50 in Western blots; Pos = Positive; Neg = Negative.

Swiss-Webster female albino mice obtained from Taconic Farms (Germantown, New York).

Serologic examination of pigs for *T. gondii*

For MAT, the antigen was prepared in the Paris Laboratory as described by Desmonts and Remington (1980). This formalin-preserved whole tachyzoite antigen was shipped by air from Paris to the Beltsville Laboratory. The MAT measures only the IgG antibodies because mercaptoethanol used in the test destroys specific as well as nonspecific IgM antibodies. Slightly different procedures are used to perform the MAT at the Beltsville and Paris laboratories. At Beltsville, mercaptoethanol is incorporated in the test as the last step to avoid prolonged exposure to fumes. At Paris, mercaptoethanol is incorporated in the serum before dilutions are made. The antibody titers using these 2 methods do not vary more than 1 or 2 dilutions. All pig sera were initially screened at Beltsville using 1:25, 1:50, 1:100, and 1:500 dilutions. Sera from pigs from lot 1 were not tested further because they all had MAT antibody titers of 1:100 or more (Table I).

Serum samples from pigs in lot 2 that were negative in the initial MAT screen were further tested, starting at a 1:10 serum dilution both at the Beltsville Laboratory and the Paris Laboratory (Table II). In addition, sera negative by MAT were also assayed at Paris using the Sabin-Feldman dye test, as described by Desmonts and Remington (1980).

For Western blot analysis, *T. gondii* oocyst proteins were prepared from oocysts collected from cat feces as described above. Oocysts were washed by centrifugation in water 4–5 times, subjected to 2 freeze–thaw cycles, then vortexed with 0.5-mm glass beads for 3- to 30-sec pulses. Protein was extracted from the oocyst preparation by the addition of reducing polyacrylamide gel electrophoresis sample buffer (2 mM 2-mercaptoethanol, 1% sodium dodecyl sulfate, 50% glycerol, pH 8.0) and submersion in a boiling water bath for 2 min. The samples were centrifuged, the supernatant was recovered, and the protein content

was estimated using a modified Bradford protein assay (BioRad, Hercules, California). Samples were electrophoresed on a 4–12% NuPage Bis-Tris gradient gel (Invitrogen, Carlsbad, California) in 50 mM, pH 8.0, 3-(*N*-morpholino)propane sulfonic acid buffer at 200 V. Proteins were electroblotted onto a Immobilon-P[®] PVDF membrane (Millipore Corporation, Bedford, Massachusetts), blocked in nonfat dry milk, and lanes were incubated overnight in the individual pig sera diluted 1:50 in 50 mM Tris-buffered, 0.85% saline. Negative control serum was obtained from *T. gondii*-free pigs from the Beltsville herd. Horseradish peroxidase-conjugated rabbit anti-swine IgG, IgA, and IgM were used as the second-step antibodies, and bands were visualized using 4-CN and peroxide as the substrate (Kirkegaard and Perry, Gaithersburg, Maryland).

Bioassay of mice for *T. gondii* infection

All mice used for bioassay were examined microscopically for *T. gondii* infection. Impression smears of lungs or mesenteric lymph nodes of mice that died were examined for *T. gondii* stages. Survivors were bled 6 wk later, and a 1:25 dilution of serum from each mouse was tested for antibodies to *T. gondii* using the MAT; mice were then killed for further examination. A 2-mm² piece of cerebrum was placed between a glass slide and a coverslip and examined microscopically for tissue cysts (Dubey and Beattie, 1998). Mice were considered infected when *T. gondii* organisms were found in their tissues. For subpassage, mesenteric lymph nodes or lungs of mice fed with oocysts were homogenized in 0.85% NaCl solution (saline) and an aliquot injected subcutaneously (s.c.) into 2 mice; an aliquot was cryopreserved for future genetic typing.

RESULTS

All pigs in lot 1 had antibodies (MAT, 1:100 or more) to *T. gondii*, and all cats fed pig hearts shed 25–810 million oocysts (Table I). All mice fed oocysts from feces of cats became ill or died 4–6 days later, and tachyzoites were found in impression smears made from their mesenteric lymph nodes. The mice inoculated with tissue homogenates of mice containing tachyzoites remained asymptomatic, and tissue cysts were found in their brains when they were killed 47 or 48 days postinoculation (p.i.); all had antibodies (MAT \geq 1:25) to *T. gondii*. All pig sera, diluted 1:50, recognized *T. gondii* oocyst proteins in the Western blot assay (Fig. 1A).

In the initial MAT screen, 9 of 25 pigs in lot 2 had no antibody to *T. gondii* in a 1:25 dilution of serum. On further examination, 6 of these pigs were seronegative at a 1:10 dilution and 3 had MAT titers of 1:20 (Table II). Antibodies were not detected in a 1:10 dilution of serum of 7 pigs in the dye test. In the Western blot assay, 18 of 25 pig sera recognized oocyst proteins (Fig. 1B).

Toxoplasma gondii oocysts were found in feces of cats fed with tissues from 21 of 25 pigs from lot 2 (Table II). The number of oocysts shed varied from 125 to 600 million. The mice fed with oocysts from 17 of 21 cats became ill or died 4–8 days p.i., and tachyzoites were found in their mesenteric lymph nodes. The mice that were fed oocysts from 3 cats that were fed tissues from pig nos. 8, 15, and 17 were apparently normal, and only a few tachyzoites were found in their lungs when killed 8 days p.i.

All mice inoculated s.c. with tissues of mice infected with *T. gondii* strains from pigs in lots 1 and 2 remained asymptomatic, and tissue cysts were found in their brains when killed on day 42 p.i.; all these mice had MAT titers of $< 1:25$ to *T. gondii*.

DISCUSSION

In this study, viable *T. gondii* was isolated from 51 of 55 market weight pigs from a known *T. gondii*-positive swine farm. This is the first such study because in the past the sources of infected pigs, verified by *T. gondii* isolation, were unknown. This is also the first demonstration of viable *T. gondii* in naturally infected market weight pigs. Although the prevalence of *T. gondii* in market weight pigs in the United States is declining drastically, the present study demonstrates that isolated foci of infected pigs still exist. Because there is no meat inspection for *T. gondii* infection anywhere in the world, there is no way to distinguish infected carcasses from uninfected carcasses. Thus, the handling or ingestion of raw or undercooked infected meat could have serious public health consequences, especially in pregnant women.

Toxoplasma gondii strains have been categorized into 3 genetic lineages, types I, II, and III (Howe et al., 1997). In the present study, mice fed with oocysts generally died, but those inoculated with tachyzoites of all 51 isolates of *T. gondii* survived for at least 2 mo. Although these strains have not been genetically characterized following Howe et al. (1997), mouse mortality data suggest that, phenotypically, these isolates are similar to type II and III strains isolated from sows in Iowa (Dubey, Thulliez, and Powell, 1995; Mondragon et al., 1998). Tachyzoites of type I strains are uniformly lethal to mice irrespective of the dose, and type I strains are considered rare in food animals except in Brazil (Dubey et al., 2002).

The isolation of *T. gondii* from 2 pigs in lot 2 without demonstrable *T. gondii* antibodies is of interest; these pigs were seronegative in the dye test, the MAT, and the Western blot. Whether these infected pigs had been infected recently and not yet developed antibodies or whether antibodies had declined because of a chronic infection is not known. Although MAT detects only IgG antibodies, the dye test detects both IgG and IgM antibodies and the Western blot detects IgG, IgA, and IgM; thus, the class of antibodies (IgG, IgA, or IgM) detected was not a factor with respect to the serological results from the 2 pigs. The most likely source of infection in these pigs is oocysts in the environment (Weigel, Dubey, Seigel, Kitron et al., 1995), and studies are in progress to understand the epidemiology of *T. gondii* on this farm. In the laboratory, pigs fed with fewer than 10 oocysts became infected and all but 1 pig developed MAT antibodies as early as 3 wk p.i. (Dubey, Andrews et al., 1996; Dubey, Lunney et al., 1996). However, in 1 pig fed with 10 oocysts, MAT antibodies were not found in a 1:25 dilution of serum when the pig was killed 69 days p.i. but viable *T. gondii* was demonstrated in its tissues. In another study, *T. gondii* was isolated from 29 of 778 pigs without MAT antibodies in a 1:20 dilution; 17 of these pigs were negative at 1:10 dilution in sera (Dubey, Thulliez, and Powell, 1995). Beverley et al. (1978) and Hejlíček and Literák (1993) also reported *T. gondii* in pigs without demonstrable dye test antibodies. Thus, with currently available serologic tests, a low percentage of *T. gondii*-infected pigs are likely to go undetected by antibody screening. Among all the serologic tests critically examined, MAT is the most sensitive and specific test used to detect *T. gondii* infection in naturally infected pigs (Dubey, Thulliez, Weigel et al., 1995).

The cats fed with pig tissues shed 25–810 million oocysts. Although cats are known to shed large numbers of oocysts after

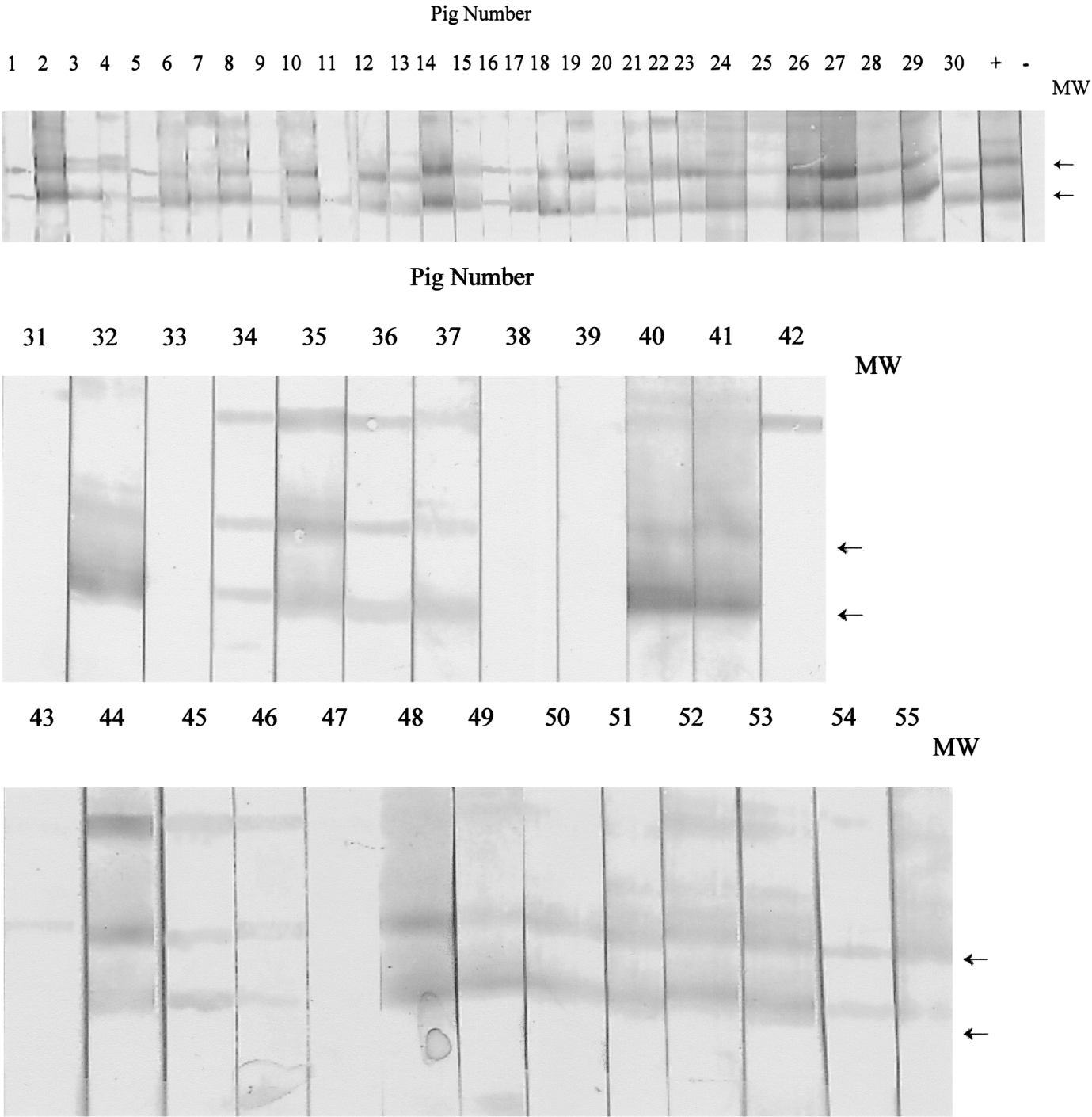


FIGURE 1. Western blot of *Toxoplasma gondii* oocyst proteins, 1:50 dilution of sera from (A) pigs from lot 1, animals 1–30, +: pig positive control serum; -: pig negative control serum, and (B) pigs from lot 2, animals 31–55. Molecular weight in kilodaltons of antigens at arrows: upper band, 45 kDa; lower band, 40 kDa.

feeding experimentally infected animal tissues (Dubey and Beattie, 1988; Dubey, 2001), the present study demonstrates that the same magnitude of oocyst shedding can occur in cats fed with naturally infected animal tissues. These data demonstrate the importance of cats in the epidemiology of *T. gondii* infection because even a few cats on a farm can contaminate the environment if they gain access to infected meat.

In the present study, pigs were not raised in confinement and had ready access to potentially *T. gondii*-contaminated environments. With the increased interest in organic farming resulting from animal welfare concerns and consumer interest in organically raised food, the issue of the increased potential for acquisition of zoonotic pathogens such as *T. gondii* must be taken into consideration.

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